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The Isolation of Simian Virus 40 Variants with Specifically Altered Genomes*

(tumor virus/deletion mutants/SV40 variants/restriction endonuclease)

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ABSTRACT **Serial passage of simian virus 40 (SV40) at high multiplicity of infection leads to the emergence of variants with deleted, substituted, and/or duplicated DNA. Individual variants have been cloned by selective complementation with temperature sensitive SV40 mutants, or nonselectively by coinfection of cells with wild-type helper virus. In each case, the presence of variants was detected by the appearance of discrete short viral genomes in infected cell lysates. Such short genomes, isolated by agarose gel electrophoresis, were shown to be specifically altered by comparing the electrophoretic pattern of their DNA fragments produced by *Haemophilus influenzae* restriction endonuclease with the pattern of fragments from parental DNA. In addition to defective variants, one infectious variant that had an additional segment of DNA within its genome was isolated.**

As with many other viruses, serial infection of susceptable cells with simian virus 40 (SV40) at high virus-to-cell ratio leads to the accumulation of virus particles with defective genomes, many of which are shorter than normal (1, 2). In the case of SV40 some of these variants contain cellular DNA sequences covalently linked to viral DNA (3). We have reported recently that after a few passages of SV40 at high multiplicity of infection, most of the progeny viruses contained genomes with deletions present in many different regions of the DNA (4). Continued serial passage led to the evolution of viruses with grossly altered genomes consisting predominantly of cellular DNA, but retaining a small portion of SV40 DNA (4, 5). Such populations of virions are therefore potential sources of deletion mutants and other types of variants that could be useful in analyzing the organization, expression, and replication of the SV40 genome as well as its interaction with cell DNA. For these purposes genetically homogeneous viruses or viral DNA are required. In this communication we describe a general method for isolating clones of SV40 variants that yields viruses with specifically altered DNA. Clones of SV40 variants have also been isolated by J. Mertz and P. Berg, and clones of defective polyoma virus have been isolated by M. Fried (personal communications).

MATERIALS AND METHODS

Viruses. The *parental stock of SV40* consisted of plaque-purified small plaque virus (no. 776) grown in the BSC-1 line of African green monkey kidney cells in minimal Eagle's medium (MEM) with 10% fetal-calf serum at a multiplicity of 0.001 PFU per cell, as detailed previously (4). The procedure used for serial passage of the parental stock has also been described (4). In the present study virus from the 3rd, 13th, and 20th passages (P3, P13, and P20) was used to isolate clones of variant SV40. *In the case of P3,* light virions were first separated from complete virus by two consecutive equilibrium centrifugations in CsCl solutions of 1.33 g/ml. The light virus stock used contained 8×10^7 PFU and 1.02 A_{260} units of virus per ml, i.e., approximately 6.5×10^{12} physical particles per ml (1). *P13 and P20* virus stocks were the lysates of the 13th and 20th passages, respectively. P13 contained 1.0×10^8 and P20 1.4×10^7 PFU/ml. *Temperature sensitive mutants of SV40* were generously supplied by Dr. Peter Tegtmeyer (6, 7). Stocks of ts A28 (an "early" mutant) and tsB4 (a "late" mutant) contained 1.5×10^7 PFU/ml.

Complementation Plaquing for the Cloning of Possible Deletion Mutants. Light virions from P3 and either tsA28 or tsB4 were used to coinfect monolayers of BSC-1 cells that had been previously adapted to 40° (and are hereafter designated BSC-40). For this purpose, from 1.8×10^4 to 1.8×10^6 physical particles of light virions in 0.05 ml of medium were added to confluent BSC-40 monolayers in 6-mm microwells, each of which contained 5×10^4 cells. Immediately afterward 2.5×10^5 PFU of tsA28 or tsB4 in 0.05 ml of medium was added. After incubation at 37° overnight, cells were transferred to 6-cm petri dishes containing 3.5 ml of medium. After incubation at 37° for 3–6 hr, approximately 10^4 freshly suspended BSC-40 cells in 1.5 ml of medium were added to each dish and immediately dispersed. After overnight incubation at 37°, the medium was replaced with an agar overlay and the dishes were shifted to 40°. Four milliliters of agar overlay was added 3 days later, and again after 6 days, the latter addition containing 0.5% neutral red. Well-separated plaques were aspirated with a pasteur pipette into 0.5 ml of MEM for further study.

For the Cloning of Defective SV40 Contained in the P13 and P20 Lysates, each lysate, which contained both infectious and defective virus, was plated at various dilutions directly on BSC-1 monolayers in 6-cm dishes at 37°. Well-separated plaques were selected for transfer to 0.5 ml of medium and subsequent assay for the presence of defective virus.

Survey of Plaques for the Presence of Deletion Variants. BSC-1 monolayers growing in 6-mm microwells were infected directly with 2 drops (about 0.05 ml) of each plaqued virus suspension. After 40 hr at 37°, the infected monolayers were

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SV40, simian virus 40; MEM, minimal Eagle's medium; *Hin* and *Hin* d, restriction endonuclease from *Haemophilus influenzae* strain d; *c*V, complementing variants; *s*V, substituted variants.

* This is publication no. 9 in a series on the genome of simian virus 40. Paper 8 is ref. 4.

rinsed, and 0.1 ml of phosphate-free MEM containing 2% serum and 40 μCi/ml of ^{32}P was added. At about 72–96 hr postinfection, the cells in each well were lysed by the method of Hirt (8). The resulting supernatants, containing viral DNA, were incubated with 10 μg/ml of heat-treated RNase A and applied directly to 1.4% (w/v) agarose (Seakem, Marine colloids) gel slabs measuring 15 × 40 × 0.16 cm. After electrophoresis in 0.04 M Tris·HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA (9) at 130 V for 16 hr, the gel was applied to x-ray film for autoradiography and the presence of rapidly migrating viral DNA species was determined.

Stocks of Variant Viruses (containing helper virus) were prepared from plaqued virus by adding 0.1 ml of the suspension to BSC-1 monolayers in 6-cm dishes, which were kept at a slight angle to increase the likelihood of coinfecting cells at one edge of the dish with defective and nondefective viruses. After 2 hr of angular incubation at 37°, 5 ml of MEM with 2% serum was added and incubation continued on a level surface at 37° until complete cytopathic effect was noted.

Large Scale Preparation of Variant DNA labeled with ^{32}P was by the method of Hirt (8) after infection of BSC-1 monolayers with variant virus stock diluted 1:4. To separate short variant DNA molecules from normal length DNA arising from the helper virus, we precipitated the DNA with ethanol after phenol extraction and subjected it to electrophoresis in 1.4% agarose. Short DNA molecules [detected by autoradiography or staining with ethidium bromide (10)], were recovered from excised gel segments by electrophoresis into dialysis sacs.

Digestion of Variant DNA by *Haemophilus influenzae* restriction endonuclease (endonuclease R·*Hin* d) and electrophoresis of digest products in acrylamide gel were carried out as previously described (11).

Electron Microscopy of Viral DNA was done by the aqueous technique of Davis, Simon, and Davidson (12).

RESULTS

Isolation of Complementing Variants. In seeking SV40 variants that had retained a substantial amount of SV40 DNA, and that could presumably express some SV40 genes (hereafter called "complementing variants" or *c*V), we used a selective technique in which plaque formation was dependent on complementation by a temperature sensitive mutant of SV40 at nonpermissive temperature. BSC-40 cells were infected with tsA28 (an "early" mutant of SV40) or with tsB4 (a "late" mutant) at a multiplicity of 5 PFU per cell, and with various dilutions of P3 light virions. Infected cells were then plated and incubated at 40°, which is nonpermissive for the growth of the ts mutants (6, 7). As shown in Table 1, infection with either ts mutant alone or with light virions alone resulted in few or no plaques, whereas coinfection with one of the ts mutants and light virus resulted in more abundant plaque formation. Many of the plaques appearing in dishes containing cells coinfected with light virions and tsB4 (but not tsA28) had more distinct borders and were more turbid than wild-type plaques. These "complementation plaques" showed sparsely stained cells within the plaque, whereas the standard plaques showed unstained cell debris.

TABLE 1. *Complementation plaques*

ts mutant	PFU/cell*	P3 light virions, particles/cell†	Infectious centers per dish
B4	5	None	0
B4	5	0.35	1
B4	5	1.0	7
B4	5	3.5	8
B4	5	10.0	38
B4	5	35.0	>75
None		35.0	19
A28	5	None	0
A28	5	0.35	0
A28	5	1.0	6
A28	5	3.5	4
A28	5	10.0	45
A28	5	35.0	>75
None		35.0	13

* PFU/cell is the ratio of input plaque-forming units of ts mutant (assayed at 32°) to the average number of BSC-40 cells present in one microwell.

† Particles/cell is the ratio of input P3 light virus particles to the average number of BSC-40 cells present in one microwell.

Detection of Deleted Genomes. To determine whether plaques contained deletion variants of SV40, we prepared viral[^{32}P]-DNA in microwells from cells infected directly with suspensions of plaqued virus, as detailed in *Methods*, and subjected the DNA to electrophoresis in agarose gel slabs. As illustrated in Fig. 1, among a large number of plaques surveyed, many yielded discrete DNA species which migrated faster than SV40 DNA in agarose gels, indicating the presence of short molecules. In a typical experiment, of 61 plaques surveyed 35 yielded one size class of short DNA, three yielded two size classes and 23 showed no short species of DNA. Length measurements by electron microscopy of short DNA molecules allowed us to correlate electrophoretic mobility with length of DNA; deleted genomes ranged from about 74% to 97% of the length of SV40 DNA.

Digestion with Endonuclease R·Hin d. To determine whether the short DNA species present in "complementation plaque" lysates were homogeneous and what segment of the genome was missing, deleted viral DNA isolated by gel electrophoresis was analysed by cleavage with endonuclease R·*Hin* d, which produces eleven specific, electrophoretically separable fragments from parental DNA (13). A digest of specifically deleted DNA should lack one or more contiguous parental fragments and should contain one new fragment. More complex alterations of the molecule, e.g., rearrangements, duplications, or insertions in addition to a deletion would result in other changes in the DNA fragment pattern. Since the order of fragments from parental SV40 DNA is known (11), one can deduce from the electrophoretic pattern of fragments present in the *Hin* digest of deleted molecules the approximate map position of the deletion. The analysis of two representative deleted genomes is shown in Fig. 2 together with the digest pattern of parental SV40 DNA (strain 776) and P3 DNA, from which the deletions were derived. In contrast to digests of parental DNA and of uncloned P3 DNA, the digest of each isolated deleted genome showed a deficiency in specific parental DNA fragments and the presence of new fragments.

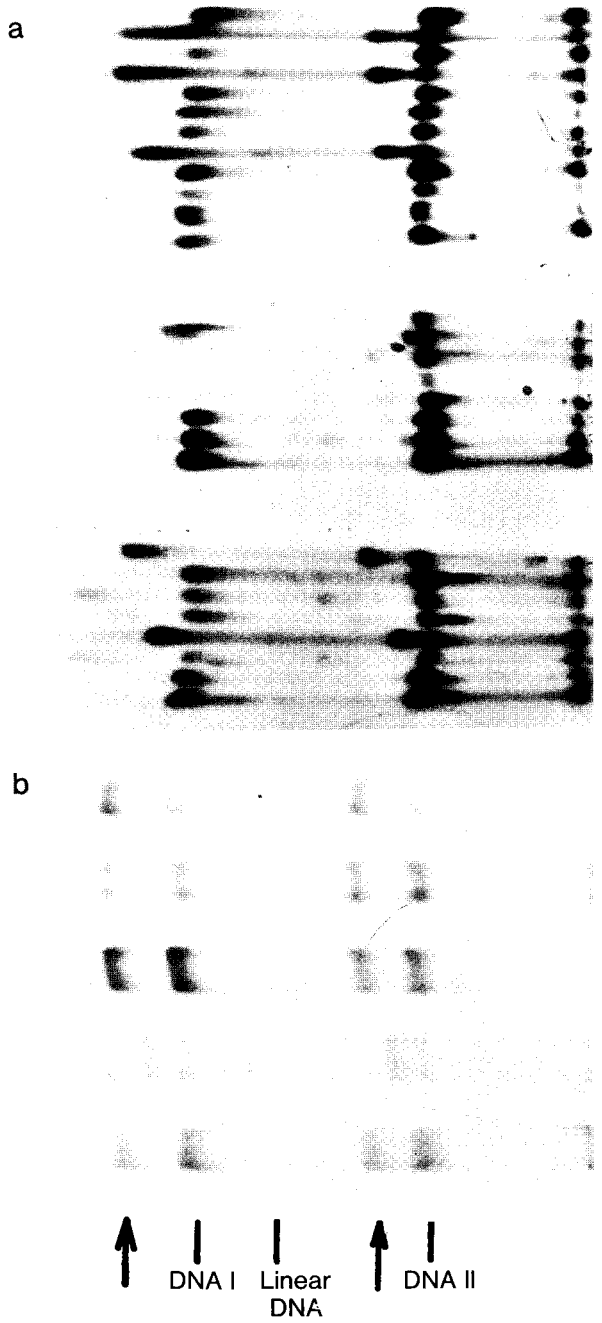

FIG. 1. Electrophoresis of variant DNA in agarose gel. (*a*) Survey of complementation plaques for variant DNA. Viral [^{32}P]DNA was labeled in BSC-1 cells in microwells as described in *Methods*, and 0.02 ml of supernatant fraction of the cell lysate was subjected to electrophoresis. Shown in the figure are representative autoradiograms of the gel slabs. (*b*) Larger scale preparation of variant DNA. Viral DNA was labeled with ^{32}P in cells infected with each of five different variant stocks, and the low-molecular-weight DNA from cell lysates was treated with phenol and precipitated with alcohol prior to electrophoresis. The positions noted for DNA I, DNA II, and linear SV40 DNA were determined by electrophoresis of parental 21S (form I), 16S (form II), and endonuclease R·*Eco* R_I digest product of form I DNA (linear DNA). The arrows point to examples of short molecules of DNA I [(*left arrow*) or DNA II (*right arrow*)]. The origin is at the right. SV40 DNA I migrated 10 cm. Not shown is the bottom two-thirds of the gel slab which contains [^{32}P]RNA fragments from the cell lysate. The bands near the origin represent oligomeric SV40 DNA.

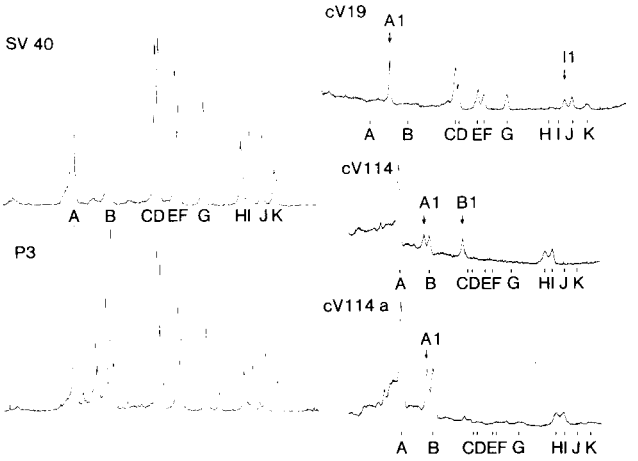

FIG. 2. Densitometer tracings of autoradiograms of ^{32}P-labeled fragments from *Hin* digestion after electrophoresis. On the left are parental SV40 DNA fragments (SV40) and fragments of DNA from third passage virus (P3). On the right are digests of *c*V19, *c*V114, and *c*V114a DNA isolated by agarose gel electrophoresis. The origin in each case is at the left of the tracing. A, B, C . . . K indicate the positions of parental DNA fragments in each gel slab.

We conclude from these findings that variants with specifically altered genomes have been isolated from the light virion population.

To locate the site of a deletion in the SV40 genome we compared the *Hin* digest pattern of each variant DNA to the *Hin* cleavage map (Fig. 3). For example, as seen in Fig. 2, *c*V19 DNA digest lacked fragments A, B, H, and I, and contained two new fragments, A1 and I1. As shown in the SV40 map (Fig. 3), *Hin* A-H-I-B form a continuous segment in the "early" region of the parental genome. We conclude that most of this segment has been deleted in *c*V19. Other alterations of this molecule have also occurred, since two new fragments (A1 and I1) are present, and in addition there are two molar equivalents of fragment C. Based on the aggregate molecular weight of fragments (11), the length of *c*V19 DNA should be

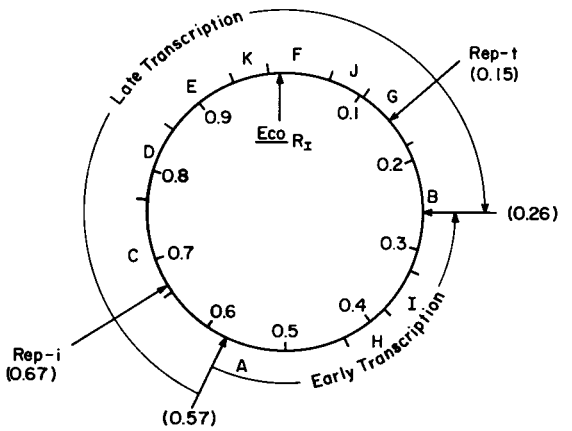

FIG. 3. The *Hin* cleavage map of the SV40 genome (11, 14). The circular map is measured from the *Eco* R_I cleavage site; designations as "early" and "late" regions of the genome are based on hybridization of "early" and "late" SV40 RNA with each *Hin* fragment (15) and the assumption that each region is a continuous segment of the genome. Rep-i is the site of initiation and Rep-t is the site of termination of DNA replication (14, 16).

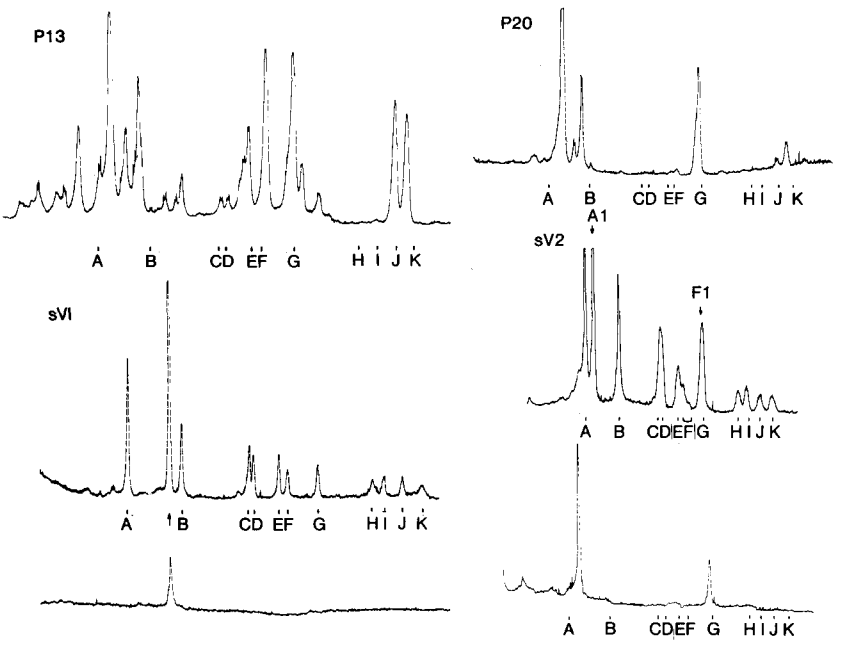

FIG. 4. Tracings of autoradiograms of ^{32}P-labeled *Hin* fragments from *s*V DNA after gel electrophoresis. The origin is at the left of each tracing. A, B, C . . . K indicate the position of parental fragments in the same gel slab. On the *left* are digests of P13 DNA (*top*), *s*V1 plus helper virus DNA (*middle*), and isolated *s*V1 DNA (*bottom*). On the *right* are digests of P20 DNA (*top*), *s*V2 plus helper virus DNA (*middle*), and isolated *s*V2 DNA (*bottom*).

85% that of parental DNA, compared to a measured mean length of 85%. These results thus indicate that *c*V19 has a deletion extending from *Hin*-B to *Hin*-A and probably an insertion resulting in duplication of *Hin*-C.

*c*V114 is an example of a variant with a deletion in the "late" region of the genome and also illustrates the usefulness of restriction endonuclease digestion to detect the presence of more than one species of deleted DNA. As shown in Fig. 2, *c*V114 lacks contiguous fragments C-D-E-K-F-J-G and has two new fragments (A1 and B1); the "early" region of the genome (A-H-I-B) appears to be present, but may be partially duplicated (A-H-I). However, more than one species of short molecules is contained in *c*V114, since the fragments are not present in stoichiometric amounts, and their aggregate molecular weight does not correspond to the measured length of *c*V114 (87% of SV40 DNA). Therefore, this variant has been recycled through the complementation selection procedure (see below), resulting in the isolation of a variant referred to as *c*V114a. As shown in Fig. 2, DNA of *c*V114a yields a simpler digest pattern than that of *c*V114, consisting of fragments A, A1, B, H, and I, fragment A being present in about two molar equivalents. The aggregate molecular weight of these fragments is 87% of SV40 DNA.

Of the ten complementation variants so far analyzed, all of those isolated by complementation with an "early" ts mutant (e.g., *c*V114) have deletions in the "late" region of the genome, and all of those isolated by complementation with a "late" ts mutant (e.g., *c*V19) have deletions in the "early" region of the genome, as defined by transcriptional analysis (Fig. 3). More detailed mapping of complementing variants can be done by combining the results of restriction endonuclease digestion with heteroduplex mapping of the genome.

Re-Cloning Variants. To strengthen the evidence that specific variants have been cloned, we re-isolated several variants by "complementation plaquing" of variant particles or DNA. For example, light virions of *c*V19, isolated by centrifugation in CsCl, were re-plaqued with tsB4 exactly as in the original isolation procedure. After infection with tsB4 or *c*V19 alone, no plaques appeared, whereas co-infection with both viruses yielded over 50 plaques per dish. All of these plaques were turbid and had distinct borders, i.e., were of the "early" complementation plaque type (see above). Of seven randomly picked plaques all yielded short genomes with identical mobility in agarose. Two of these were digested with endonuclease R·*Hin* d and gave an electrophoretic pattern of *Hin* fragments identical to that of the original *c*V19 (data not shown). We conclude that selective complementation does yield clones of variant SV40 and that at least some of these variants are stable.

Isolation of Variants with Substituted DNA. As indicated earlier, continued passage of SV40 at high multiplicity leads to the evolution of new species of viral DNA containing primarily nonrepetitive cellular DNA sequences and little SV40 DNA (4). We wanted to isolate clones of these substituted variants (*s*V) in order to determine their precise structure. Since such variants may not be able to express any SV40 genes, they were isolated nonselectively. For this purpose late passage lysates (P13 or P20), which contain defective and nondefective virus, were plated on BSC-1 cells and random, well-separated plaques were selected for growth of stock virus. To survey these stocks for the presence of altered virus, we prepared viral [^{32}P]DNA from infected cell lysates, as detailed in *Methods*, and examined the [^{32}P]DNA samples in two ways: (*1*) by electrophoresis in agarose to detect short genomes, and (*2*) by digestion with endonuclease R·*Hin* d followed by electrophoresis of DNA fragments. Among a large number of plaques surveyed, several yielded virus with short genomes and/or discrete *Hin* fragments not present in digests of parental DNA alone. Two examples of this type of variant genome are presented in Fig. 4. As seen in the figure, when the

short DNA molecules recovered from agarose gels were digested, only one or two fragments were detected, in contrast to uncloned P13 and P20 virus DNA, from which the respective variants were derived. These results indicate that specific variants which contain grossly altered genomes have been isolated.

In the examples shown in Fig. 4, the fragments present in *Hin* digests total only 17% (*s*V1) and 27% (sV2) of the length of parental viral DNA as estimated from electrophoretic mobility (11). Since *s*V1 is approximately 85% and sV2 about 80% as long as SV40 DNA, as estimated by mobility in agarose and length measurements, each of these genomes must be made up of a tandemly repeating unit of DNA. Unpublished experiments by T. N. H. Lee and D. Nathans indicate that variants of this type contain cell DNA, and the repeating unit also contains a small segment of SV40 DNA that includes the site of initiation of SV40 DNA replication.

Isolation of an Infectious Variant with Added DNA. One plaque isolated nonselectively from P13 lysate contained virus whose DNA yielded a novel *Hin*-C fragment. The variant C fragment was 12.5% of the length of SV40 DNA compared to 10.5% for the parental fragment, as estimated by electrophoretic mobility. After serial plaque purification, only virus with variant DNA was obtained, indicating that this variant was infectious. (This isolate has been labelled infectious variant 1 or *i*V1.) The identity of the inserted or duplicated DNA segment is as yet undetermined.

DISCUSSION

In this communication we have described a general method for cloning lethal variants of SV40, a method that should be applicable to other viruses as well. Selective complementation with conditional mutants has been used to isolate variants that can express some SV40 genes, and nonselective coinfection with wild-type virus has been used to isolate variants that retain little SV40 DNA. In each case, separation of the defective variant from the "helper" virus depends on a difference in size of the two viral genomes. This difference has allowed separation of the DNAs by electrophoresis in agarose and of the virions by equilibrium centrifugation in CsCl. The method should yield variants with defects in any portion of the genome, with the exception of regions needed for *cis* functions involved in DNA replication.

SV40 with specific deletions of the genome will be useful in mapping viral genes, in identifying SV40-coded proteins, and in physiological studies of SV40 development and tumorigenesis. As illustrated in this communication, rapid localization of a given deletion is possible by comparing the restriction endonuclease products of deleted DNA with those of parental DNA and relating these differences to the cleavage map of the genome. More precise mapping of each variant can be accomplished by combining the results of heteroduplex mapping and restriction endonuclease digestion, and the sites of alteration can be correlated with biological properties of the variants.

A potential difficulty in the use of high multiplicity passage virus as a source of mutants is the possibility that several alterations of a deleted genome, which would affect its function, may be present, as already indicated by the results reported in this paper. Probably due to selective pressure there appears to be rather frequent duplication of the initiation site for DNA replication located in fragment *Hin*-C (Fig. 3). For this reason we have constructed SV40 genomes containing specific deletions by excising defined segments from wild-type viral DNA, using suitable restriction endonucleases. Some of these excisionally deleted genomes have been cloned by complementation with ts mutants of SV40, and have been shown to contain the expected deletions (C.-J. Lai and D. Nathans, manuscript in preparation).

The isolation of clones of variant viruses containing cellular DNA and a small amount of SV40 DNA (substituted variants) provides an opportunity to examine the site(s) of recombination between SV40 DNA and host cell DNA, which may be related to the integration of the SV40 genome into host chromosomes. In addition, some of the variants have been shown to contain the region of SV40 DNA at which DNA replication begins (14) (T. N. H. Lee and D. Nathans, unpublished observations). Since these variant molecules consist of tandemly repeated segments of DNA, they contain several copies of the initiation signal, as postulated earlier (4), which could explain their evolution during serial passage. Similar repetitious variant DNA containing only SV40 sequences has been isolated and characterized in detail by G. Khoury, G. C. Fareed, K. Berry, M. A. Martin, T. N. H. Lee and D. Nathans (manuscript in preparation).

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